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Patents ADP number <i>(if you know it)</i>	473587003		
If the applicant is a corporate body, give the country/state of its corporation			
4 Title of the invention	CD28 BINDING PROTEIN		
5 Name of your agent <i>(if you know one)</i>	MICHAEL J. STOTT (SEE CONTINUATION SHEET)		
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11. I/We request the grant of a patent on the basis of this application

Signature: Michael J. Stott

19 August 1998

AGENT FOR THE APPLICANTS

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The stimulation of T lymphocytes by antigen-presenting cells is believed to require activation of two groups of intracellular signalling pathways (Weiss and Imboden, 1987; Allison, 1994). One signal is derived from the ligation of the T cell receptor complex by antigen in association with MHC molecules, resulting in the activation of a number of intracellular protein tyrosine kinases (reviewed in Peri and Veillette, 1994), principally p56lck and ZAP70. These events can lead to the cell entering an activated or a non-responsive state (Quill and Schwartz, 1997; Mueller et al, 1989a; Mueller et al, 1989b; DeSilva et al, 1991; Harding et al, 1992), depending on the presence or absence of a second, or costimulatory, signal resulting from the ligation of various accessory molecules on the T cell surface.

Although a number of cell surface molecules have been shown to possess costimulatory activity, notably CD2, VLA4 and LFA-1 (van Seventer et al, 1991; June et al, 1994a); the best characterised and most potent stimulus arises from the interaction of CD28 on the T cell surface with its counter-receptors CD80 (B7-1; Freeman et al, 1989) and CD86 (B7-2; Freeman et al, 1993a,b; Azuma et al, 1993; Caux et al, 1994) on APC (Harding et al, 1992; June et al, 1994; Freeman et al, 1993a,b; Galvin et al, 1992; Judge et al, 1995). Indeed, exposing peripheral blood CD4-positive T cells to a TCR stimulus such as immobilised anti-CD3 together with CD80 or CD86 is sufficient to drive proliferation (Freeman et al, 1993a,b; Linsley et al, 1991a). Several anti-CD28 monoclonal antibodies also possess stimulatory activity (reviewed in June et al, 1990; June et al, 1994b).

In addition to CD28, many T cells populations also express a second ligand for CD80 and CD86: CTLA4. This molecule shares significant primary structure homology with CD28 (Linsley et al, 1991b). CTLA4 has been shown to bind CD80 and CD86 with a higher affinity than does CD28, a difference of at least 20-fold (Linsley et al, 1991a,b; Freeman et al, 1991; Peach et al, 1994). Activation of CTLA4 appears to produce an inhibition of T cell activation, opposing the stimulatory signal from CD28 (Walunas et al, 1996).

Thus, the operation of the CD28 and CTLA4 pathways is interlinked, and the final outcome of a T cell activation attempt may depend on the relative balance and interplay between the CD28 and CTLA4 signals. To reflect this interlinking, the term 'B7 costimulatory system' is used herein to refer to the integrated system encompassing both CD28 and CTLA4 signals.

The therapeutic potential of agents that manipulate the B7 costimulatory system has been well recognised, and their use has been demonstrated in auto-immune disease, cancer and other conditions of immune dysfunction.

Foremost amongst these agents is the soluble receptor CTLA4-Ig. This consists of the extracellular domain of CTLA4 genetically fused to immunoglobulin constant region, as disclosed in US 5434131 and WO 93/00431. This molecule binds with high affinity to CD80 and CD86, and prevents their association with CD28 and CTLA4 on T cells. It therefore acts as a competitive antagonist of the B7 costimulatory system. Its efficacy has been demonstrated in a wide variety of systems, both *in vitro* and *in vivo* (Linsley et al, 1992; Corry et al, 1994; Wallace et al, 1994; Finck et al, 1994; Ronchese et al, 1994; Perrin et al, 1995), where it has marked beneficial effects in many manifestations of autoimmune disease.

Antibodies which bind to CD80 and/or CD86 and block interactions with CD28 and/or CTLA4 are also well known. These too have been shown to have beneficial therapeutic utility in models of autoimmune diseases (see for example Keane-Myers et al, 1998; Katayama et al, 1997).

Antibodies which bind to and block the CD80/CD86-binding activity of CTLA4 have also been described. These modulate the B7 costimulatory system by preventing the negative CTLA4 signal from antagonising the stimulatory signal produced when CD28 binds CD80 or CD86. The net result of this modulation is an enhancement of T cell responsiveness. Their utility in the treatment of cancer has also been shown (see for example, Lach et al, 1996).

Antibodies which bind to either CD28 or CTLA4, and provide an activating signal in the absence of ligand are also known. Such antibodies which activate CD28 generally have a net stimulatory effect (see for example Ledbetter et al, 1990), which may result in cellular proliferation and the production of cytokines. Antibodies which activate CTLA4 generally have a net inhibitory effect, and may induce inactivity, anergy or apoptosis (see for example Walunas et al, 1996).

However, all of these approaches to the modulation of the B7 costimulatory system rely on large protein molecules which have many disadvantages as therapeutic agents. Generally they must be administered by injection or infusion as they are poorly orally bioavailable. Furthermore, they may be recognised by the immune system as foreign protein and therefore made the target of an undesired immune response. This may neutralise the biological activity of these polypeptides, or may have deleterious consequences for the host, such as the development of allergy or anaphylactic shock.

As a consequence, a preferred approach to the manipulation of the B7 costimulatory system is through the development of non-polypeptide agents which modulate the activity of the CD28 or CTLA4 pathways. Preferably such agents are easily synthesised small molecular weight chemical entities. These may modulate the binding of CD28 or CTLA4 by CD80 or CD86 in a manner similar to the polypeptide agents described above.

Alternatively, some such agents may permeate into the cell where they may act to modulate the expression or function of proteins involved in the pathways by which CD28 and CTLA4 affect cellular function. Previous investigations have identified a number of signalling molecules as forming part of the CD28 or CTLA4 signalling pathways, and these could be considered as targets for the activity of agents designed to modulate the B7 costimulatory system. For example, the p85 subunit of PI3-kinase and Grb2 have been shown to associate with CD28 following activation (reviewed in Ward, 1996). CTLA4 has been shown to associate with p85, SHP2, and AP50 (Schneider et al, 1995; Marangere et al, 1996; Zhang and Allison, 1997).

However, these molecules are present in cell types other than T cells and are also components of signalling pathways outside the B7 costimulatory system. Therefore, putative therapeutic approaches that manipulate such signalling molecules have substantial potential for unwanted and undesirable side effects by modulating other pathways. As a more advantageous strategy, it would be better to manipulate the expression or function of a protein which is expressed principally in T cells and which interacts specifically with CD28 or CTLA4.

Summary of the invention

Accordingly, in one aspect of the present invention, the nucleotide and deduced protein sequence of a protein with exactly these desirable properties is provided. This protein is herein termed GRIP.

In another embodiment of the invention, vectors enabling the expression of GRIP in both eukaryotic and prokaryotic cells are also provided.

In another aspect of the invention, antibodies are provided that specifically recognise GRIP. These antibodies are useful in the detection of GRIP, and hence in the identification of

diseases or abnormal cellular states in which modulation of GRIP function or expression may be beneficial.

To facilitate the identification of therapeutic agents which modulate the function of the B7 costimulatory system, other embodiments of the invention provide simple biochemical and immunological assays for the detection of the interaction between GRIP and CD28 and other proteins. It will be apparent to those skilled in the art that these assays may be employed to identify molecular entities capable of disrupting this interaction.

In another aspect of the invention, a PCR-based assay is provided that enables the specific detection of mRNA encoding GRIP from total RNA derived from cells that express GRIP. It will be apparent to those skilled in the art that this assay may be used to identify molecular entities capable of modulating the expression of GRIP mRNA and hence GRIP protein and therefore affecting the operation of the B7 costimulatory system.

Detailed description of the invention

Throughout the following examples of the invention, use is made of various widely known and practised techniques in molecular and cellular biology. Practical details of these may be found in a number of textbooks including Sambrook et al, 1989. Unless otherwise stated, PCR reactions were performed using AmpliTaq enzyme (Roche) in reaction mixes containing buffer and nucleoside triphosphates at the recommended concentrations, and primers at a concentration of 1 μ M each. Thermal cycling was performed according to the following general scheme: 5 minutes at 95°C followed by a number of cycles usually between 10 and 50, each made up of 1 minute at 95°C, 1 minute at an appropriate annealing temperature, most often 50°C, and 1-2 minutes at 72°C. Optionally a further 5 minute incubation at 72°C may be added. The precise times, temperatures and number of cycles may be altered as widely known by those skilled in the art to optimise the reaction yield for the particular thermal cycler, reaction tubes and other practical variables that may apply to any given laboratory. Numbered sequence positions that refer to amino acid residues in human CD28 are according to the scheme adopted by Barclay et al (1997), which is based on the amino acid sequence of full length mature human CD28. Amino acid sequences or designations may be given in either the one letter code, or the three letter code.

Example 1: Cloning of plasmids

A number of modifications to previously published plasmids were undertaken to facilitate cloning. The polylinker of the plasmid pYTH9 (Fuller et al, 1998) was modified by standard molecular biology techniques to insert an in-frame BssHII site. The sequence of the modified polylinker is shown in Figure 1: this plasmid is termed pYTH9/BssHII. A similar procedure was undertaken using the plasmid pAS1CYH2 to create pAS1CYH2/BssHII (Figure 2). A similar procedure was also performed to alter the reading frame of the BamHI site in plasmid pACT2 to create pACT2/BamHI (Figure 3). pAS1CYH2 and pACT2 are standard yeast two-hybrid vectors (Clontech MATCHMAKER kit; Clontech, Palo Alto, CA; Harper et al, 1993)

Four overlapping oligonucleotides (CC205, CC206, CC207 and CC208) which together encode the entire cytoplasmic domain of human CD28 (Figure 4) were mixed in the molar ratios 10:1:1:10 respectively and then subjected to a PCR reaction to generate the full length cytoplasmic domain of human CD28 tagged with BssHII and NotI restriction sites in the correct reading frame for preparing fusion proteins with GAL4BD.

The resulting PCR product was digested with BssHII and ligated into a variant of pUC18 into which a unique BssHII cloning site had previously been inserted by standard molecular

biology techniques. A clone of the expected sequence named pxC28Y was identified by fluorescent dye-terminator sequencing. pxC28Y was digested with BssHII and NotI to liberate the CD28 insert. This fragment was purified by preparative agarose gel electrophoresis and then inserted into BssHII-NotI-cut pYTH9/BssHII. A correct clone of the resulting plasmid (pY3H8Y) was identified by sequencing as above. This plasmid therefore encodes a fusion protein consisting of the binding domain (BD) of the GAL4 protein fused in frame to the cytoplasmic domain of human CD28. The same strategy was used to transfer a BssHII fragment bearing the CD28 sequence from pxC28Y into BssHII digested pAS1CYH2 to create plasmid pCD28Y.BD, also encoding a GAL4BD-CD28 fusion protein.

A sequenced clone of human p85 α (Genbank M61906) was used as a template in a PCR with primers CE14 and CE15. The resulting product was digested with BamHI and EcoRI, purified by preparative gel electrophoresis and ligated into BamHI-EcoRI cut pACT2/BamHI. A clone of the correct sequence was identified as above, and termed p85SH2C.AD. This construct encodes a fusion protein consisting of the GAL4 activation domain (AD) fused in frame to a portion of human p85 α including the C terminal SH2 domain (GAL4AD-p85SH2).

pY3HY8 was linearised by digestion with XbaI, and transfected into *Saccharomyces cerevisiae* Ylck4.1 as described (Fuller et al, 1998). Single clones growing in media lacking tryptophan were selected and analysed for the presence of the gene encoding the GAL4BD-CD28 fusion protein. Yeast chromosomal DNA was isolated as described (Fuller et al, 1998) and samples subjected to PCR using primers CC205 and CC208. Several clones showing a positive signal in the PCR were cultured and cellular protein extracted by boiling in SDS-PAGE sample buffer. Samples were then subjected to SDS-PAGE, electrophoretically transferred to nitrocellulose, and probed for the presence of GAL4BD-CD28 fusion protein using a monoclonal antibody against GAL4BD (Santa Cruz cat #SC510). Bound antibody was visualised using a chemiluminescent method as described by the manufacturer (SuperSignal; Pierce). A yeast clone expressing a fusion protein of the expected size was chosen for further work and termed Y8.

Example 2: Identification of GRIP

To identify novel proteins binding to CD28, a variant of the yeast two hybrid technique was employed (Fuller et al, 1998). In the yeast two hybrid, use is made of the fact that the GAL4 transcription factor contains two domains: the activation domain (GAL4AD) and the binding domain (GAL4BD). Formation of a close physical complex between these two domains reconstitutes a transactivating activity which can be employed to activate expression of suitably prepared reporter genes. These two domains can be expressed separately as fusion proteins with heterologous proteins, in such a fashion that if the heterologous fusion partners form a complex with each other, the two GAL4 domains are brought into apposition and become active. If the fusion partners do not interact, there is no such activity. Hence, this system forms a powerful tool for the determination of whether two arbitrary proteins interact. This principle is well known in the field, and there are a number of variants and applications which have been described (see for example Fuller et al, 1998).

Yeast strain Y8 contains the GAL4BD-CD28 fusion protein and also catalytically active murine Lck derived from its parent Ylck4.1 (Fuller et al, 1998). The Lck gene is expressed under a regulated promoter such that it is expressed in the absence of methionine in the culture medium and repressed by the presence of 2mM methionine (Fuller et al, 1998). Since the CD28 cytoplasmic domain contains a residue (corresponding to tyrosine 173 in mature human CD28) which is a substrate for Lck (see for example King et al, 1997), it was predicted that in the presence of the tyrosine kinase in the yeast, the fusion protein would become phosphorylated at this site. Furthermore, it is also known that CD28 bearing phosphotyrosin at this site is bound by the p85 subunit of PI3 kinase, via its SH2 domains (see for example Raab et al, 1995). By analogy with previous experience in this system, we predicted that association of tyrosine phosphorylated GAL4BD-CD28 fusion protein with GAL4AD-p85SH2

would recreate GAL4 transactivating activity, causing expression of the LacZ and HIS3 reporter genes present in this yeast strain (Fuller et al, 1998). No transactivation would be predicted in the absence of Lck, as SH2 domains require phosphotyrosine for their binding site.

Yeast clone Y8 expressing the GAL4BD-CD28 fusion protein was transfected with either plasmid p85SH2C.AD or plasmid pACT2 and transformants selected on media deficient for tryptophan and leucine (Met⁺ media) or media deficient for tryptophan, leucine and methionine (Met⁻ media). Samples of growing yeast were assayed for LacZ activity as described (Fuller et al, 1998). Only Y8 yeast transfected with p85SH2C.AD and growing on Met⁻ media showed LacZ activity, indicating that GAL4BD-CD28 interacts with GAL4AD-p85SH2 but not GAL4AD. Furthermore, this interaction was contingent on the coexpression of catalytically active Lck.

These results are exactly as predicted, and indicate that this yeast system accurately recreates the previously observed regulated interaction between CD28 and p85. Based on this validation, we then used the Y8 yeast to screen a pACT2 GAL4AD fusion protein expression library for potential novel binding partners for phosphorylated CD28. This was done essentially as described (Fuller et al, 1998), using the same library. A number of clones were obtained that expressed LacZ activity only when the Lck gene was expressed. Plasmid DNA encoding the GAL4AD fusion was recovered from each clone and sequenced by fluorescent dye-terminator sequencing. One clone, termed Y8.41, yielded a plasmid herein termed pY3HY8.41 which contained a sequence previously unknown, and was selected for further analysis.

Sequence analysis of pY3HY8.41 revealed an open reading frame in addition to the vector-derived sequence. The deduced amino acid sequence contained motifs characteristic of SH2 and SH3 domains. The 5' end of the insert did not encode a methionine in the correct context to be a start residue, and also contained sequence homologous to a C terminal fragment of an SH3 domain. We therefore postulated that the pY3HY8.41 clone was incomplete, and that further 5' sequence was required to obtain the full coding sequence.

The nucleic acid sequence was used to screen the Genbank database, and two highly similar partial sequences of previously unknown function identified. These were found to overlap the 5' end of the pY3HY8.41 sequence, allowing the preparation of a longer consensus sequence. Upon analysis, this sequence was found to contain both a methionine in a context close to that required for optimal translation initiation (Kozak, 1984) and also the missing portion of the predicted SH3 domain. Based on this consensus sequence, PCR primers CE65 and CE66 were designed to amplify the full length coding sequence for the pY3HY8.41 insert. Total RNA was prepared from 10⁶ actively growing Jurkat T cells using the Promega SV Total RNA Isolation kit according to the manufacturer's instructions. Aliquots of the RNA were then subjected to RT-PCR using primers CE65 and CE66 and the Promega Access kit according to the manufacturer's instructions. The resulting PCR product was digested with BssHII and NotI, purified by preparative gel electrophoresis and ligated into appropriately prepared pYTH9/BssHII. Clones containing inserts of the expected size were analysed by fluorescent dye-terminator sequencing, and a complete DNA sequence obtained (Figure 5).

This sequence matches the sequence obtained from pY3HY8.41 and also the consensus sequence prepared using the combination of pY3HY8.41 and the partial sequences from Genbank. Analysis of the deduced protein sequence reveals the presence of two putative SH3 domains, one at the N terminal encoded by cDNA residues 13 to 156, and one at the C terminus of the protein encoded by residues 832 to 990. Between these lie a putative SH2 domain (residues 172 to 441) and another domain of unknown function (residues 442 to 831), rich in proline, glutamine and histidine residues. This latter domain we term the 'insert domain'. The schematic format of the full length protein is therefore N-SH3-SH2-Insert-SH3-C. It will be apparent to those skilled in the art that the boundaries of the domains delineated above are provided simply for ease of reference, and that fragments of sequence inside these

boundaries may be deleted, or sequence outside these boundaries added, without detracting from the properties of the domain.

A further search of the sequence databases revealed that the most closely-related known proteins are Grb2 (Genbank M96995; Lowenstein et al, 1992) and Grap (Genbank U52518; Feng et al, 1996). Both of these have the schematic structure N-SH3-SH2-SH3-C, ie they lack a domain homologous to the insert domain. We therefore named the novel full length gene GRIP, standing for Grb2-Related with Insert Protein. The plasmid containing the complete insert, we termed pGRIP.BDI. Since the insert domain is unique to GRIP, it is likely to possess interesting and particular properties not present in other proteins.

Our analysis of the GRIP cDNA sequence indicates that it should encode a protein of approximate molecular weight 38 kilodaltons. Those skilled in the art will know that this may be subsequently altered by post-translational modifications such as phosphorylation, myristylation, palmitoylation, glycosylation, proteolytic cleavage for example. There does not appear to be a sequence consistent with signal sequences which direct the export of proteins from the cell. There are also no sequences with the characteristics of membrane-spanning stretches of polypeptide. GRIP is therefore most likely to be a cytoplasmic protein, like its closest relatives Grb2 and Grap. The SH3 and SH2 motifs are characteristic modules implicated in protein:protein interactions, and suggest that GRIP is one of a class of proteins known as adapter proteins. Such proteins play critical roles in a wide range of signal transduction pathways (reviewed in Birge et al, 1996). The SH3 domain interacts with proteins containing sequences of the general format Pro-Xxx-Xxx-Pro (where Pro is proline and Xxx is any amino acid residue, also sometimes denoted PXXP sequences using the one letter amino acid code) (reviewed in Musacchio et al, 1994). SH2 domains interact with phosphotyrosine containing motifs (reviewed in Schaffhausen, 1995) generated by the activity of protein tyrosine kinases upon substrate proteins. The insert domain may have a similar role in protein:protein interactions, or it may have an enzymic function or a nucleic acid binding function or it may have a role in determining the three-dimensional disposition of other parts of GRIP or it may have some other function.

Since a major function of such adapter proteins is to associate with other proteins and to modulate their function or localisation, it will be apparent that either other chemical entities or mutations in GRIP which modulate the ability of GRIP to bind other proteins may be employed to therapeutic effect to modulate the function of pathways in which GRIP plays a role. These may target individual domains in GRIP, individual binding sites in or for GRIP, or may affect other portions of GRIP, or even the entire protein.

It should be noted that sequences other than that laid out in Figure 5 may also be determined, perhaps revealing deletions, additions or mutations in any given GRIP cDNA clone or mRNA or genomic DNA sample. These changes may or may not affect the deduced amino acid sequence of the individual GRIP clone. Such variant sequences that are substantially the same as GRIP do not depart from the scope of the present invention. Such variants that are substantially the same will generally have amino acid similarities to GRIP which may exceed the level of 99% or 95% or 90% or 85% or 80% or 70% or 50%. Furthermore, there may also be GRIP sequences in which one or more exons have been removed, replaced or added by alternative splicing. These too are encompassed by the present invention.

Example 3: Localisation of GRIP expression

Since the related protein Grb2 is ubiquitously expressed, we were interested to examine the tissue distribution of GRIP expression. We prepared primers CE71 and CE72 which hybridise to residues 430 to 453 and 802 to 825 respectively in the sequence of Figure 5. These were employed in PCR using as template cDNA prepared from mRNA extracted from a variety of normal adult tissues. cDNA obtained from skin, brain, liver, colon, skeletal muscle, testis and lung was obtained from Invitrogen (Discovery Line; Invitrogen;). cDNA libraries prepared from normal human lymph node and spleen were a kind gift of Dr E Zanders, GlaxoWellcome, UK.

In parallel control reactions, we also employed primers CE102 and CE103 specific for a portion of the GAPDH message (a ubiquitously expressed gene the presence of whose cDNA in a library is widely used as a marker for both success of a PCR reaction and of the cDNA preparation).

PCR reactions contained each primer at a final concentration of 1 μ M, 1.25 units of AmpliTaq (Roche), 1 μ l of cDNA and nucleoside triphosphates and buffers at the concentrations recommended by the enzyme supplier (Roche). The reactions were placed in a thermal cycler (Trio, Biometra) and subjected to 5 minutes at 95°C followed by a number of cycles, each made up of 1 minute at 95°C, followed by 1 minute at 50°C, followed by 1 minute at 72°C. Reactions with GRIP primers used 45 cycles, those with GAPDH primers, 35 cycles.

At the end of the reaction, 10 μ l samples of each reaction were analysed by agarose gel electrophoresis. The results are shown in Figure 6. All of the GAPDH reactions showed strong signals, indicating successful cDNA preparation and ubiquitous expression of GAPDH. In contrast, specific signals were only observed in two of the GRIP reactions: those using spleen and lymph node cDNA as template. These data indicate that GRIP expression is confined to lymphoid tissue.

Since lymphoid tissue contains many cell types (T cells, B cells, monocyte lineage cells for example), we next prepared total RNA as described previously from Jurkat (a T cell line), OZZ and MAW (two B cell lines) and Thp1 (a monocyte lineage cell line). RT-PCR reactions were prepared using the Promega Access RT-PCR kit according to the manufacturer's instructions (Promega, Madison, WI). Each reaction contained 1 μ M final concentration of each of two primers, either CE71 and CE72 for GRIP or CE102 and CE103 for GAPDH. Cycling was performed according to the manufacturer's instructions, using 45 cycles for each reaction. Upon completion, 10 μ l samples were analysed by agarose gel electrophoresis. The results are shown in Figure 7, and demonstrate that GRIP mRNA is only expressed in the T cell line. We therefore believe GRIP expression to be primarily confined to T cells.

It will be apparent to the person skilled in the art that these or similar primer sequences and PCR or RT-PCR reactions (or other amplification or hybridisation technologies, as well known and widely practised), may be employed to specifically determine the level of GRIP mRNA in the manner of a diagnostic kit. GRIP mRNA overexpression may be associated with conditions of inappropriate immune system activity, such as autoimmune diseases like rheumatoid arthritis, psoriasis, allergic asthma. Similarly, GRIP mRNA under-expression may be associated with conditions of insufficient immune system activity such as cancer, or immunosuppression.

Moreover, although for our convenience we have used particular methods for the detection of GRIP mRNA or cDNA, it will be obvious to those skilled in the art that suitable probe sequences may be derived from the teaching presented herein to allow detection of GRIP mRNA, cDNA, genomic DNA derived from human or other species by standard methods including Northern blotting or Southern blotting. Also, under appropriate experimental conditions, the same may be used to enable the detection, characterisation or purification of polynucleotide sequences closely related to GRIP by virtue of the possession of sufficient homology to allow selective hybridisation to one or more probe sequences.

Example 4: Regulation of GRIP expression

To determine whether GRIP expression was modulated by external stimuli, we isolated and purified peripheral blood CD4-positive T lymphocytes as previously described (Ellis et al, 1996). These cells were stimulated for varying periods of time with activating antibodies against CD3 (mAb OKT3) and CD28 (mAb 9.3) immobilised on a plastic tissue culture well. After the specified period of time, the cells were immediately lysed and total RNA prepared from the lysates as described above. Two cohorts of cells were not lysed at the end of their

period, but instead were incubated with ^3H -thymidine as part of a standard thymidine incorporation assay for cell proliferation as a control for successful stimulation. One cohort was not treated with the activating antibodies, the other was exposed to them for 48 hours. The results of this are shown in Figure 8, and clearly show that the activating antibody treatment was successful in activating the cells to proliferate.

Samples of each total RNA preparation were then analysed by RT-PCR for GRIP and GAPDH mRNA levels essentially as described above, except that in the GRIP RT-PCRs, primers CE65 and CE66 were used, and the reactions received 50 cycles. The results are also shown in Figure 8. These data demonstrate that while GRIP mRNA is present in unactivated resting cells, its level markedly increases upon cell activation, in excess of a small general increase in cell mRNA as evidenced by an increase in the GAPDH signal.

One skilled in the art can readily see how this assay may be adapted and utilised to search for agents which specifically modulate the levels of GRIP mRNA in some desired fashion, perhaps increasing the amount of GRIP mRNA or decreasing the amount of GRIP mRNA. Furthermore, by utilising the RT-PCR for GAPDH mRNA, it is possible to distinguish agents which specifically act on GRIP mRNA levels, rather than more generally modulating mRNA levels within the cell. Such agents may include antisense RNA or DNA, triplex-forming oligonucleotides, ribozymes and similar agents well known to those in the field.

Example 5: Expression of GRIP in eukaryotic cells

To confirm that the full-length GRIP insert did indeed encode a translatable protein, it was transferred to a eukaryotic expression vector. A small amount of plasmid pGRIP.BDI was used as template in a PCR reaction with primers CE65 and CE79 which attach a number of restriction sites. The resulting PCR product was digested with BamHI and EcoRI and ligated into appropriately prepared pcDNA3.1HisC (Invitrogen; Holland). This plasmid provides two short peptide tags (the Xpress tag and the His6 tag) at the N-terminus of the inserted protein which may be used for identification or purification of the protein. The resulting plasmid, pGRIPFL.His, was analysed by fluorescent dye-terminator sequencing, and found to have a small deletion, creating a spurious BamHI site within one of these tags, such that the downstream GRIP insert was thrown out of the reading frame.

To correct this error, a pair of complementary oligonucleotides CE106 and CE107 were designed, annealed together, and then cloned into pGRIPFL.His at the spurious BamHI site. The resulting plasmid, pGRIPFL.Fix was analysed by sequencing as above, and found to have the predicted structure. The sequence of the 5' end of this construct is shown in Figure 9, illustrating the nature of the junction with the vector-encoded tag sequences.

To prepare this construct in a form suitable for expression in eukaryotic cells, a sample of pGRIPFL.Fix was then used as the template in a PCR using primers CE108 and CE109. These attach a consensus translation initiation sequence upstream of the initial methionine codon, and also attach EcoRI cloning sites to the ends of the insert. The resulting PCR product was digested with EcoRI and cloned into suitably prepared pCI (Promega). pCI contains a promoter and other elements required for expression of inserts in eukaryotic cells. Clones were analysed by restriction digestion, and one showing the predicted pattern of restriction sites taken for further analysis. The structure of this plasmid, pGRIP-X was then confirmed by fluorescent dye-terminator sequencing. A similar construct, pGRIP-H, bearing the HA-tag in place of the Xpress and His6 tags was prepared in a similar fashion, using primer CE110 in place of CE108.

Samples of pGRIP-X, pGRIP-H, empty parental pCI vector or pcDNA3.1HisLacZ (a control plasmid containing an expressible gene for another protein tagged with the Xpress epitope; Invitrogen) were transfected into COS1 cells using lipofectamin according to the manufacturer's protocol (Gibco BRL). After 48 hours the cells were lysed and prepared for SDS-PAGE according to standard protocols. Samples were separated on 8-16% acrylamide

gradient g ls (Novex), blotted to nitrocellulose and then probed for the presence of the Xpress tag using an anti-Xpress mAb (Invitrogen) according to standard Western blotting protocols. Bound antibody was visualised as described above. The results are shown in Figure 10, and reveal that pGRIP-X specifically directs the expression of a circa 45kDa protein containing the Xpress tag. This is in good agreement with the predicted molecular weight of the GRIP protein once the size of the tags has been accounted for. These data demonstrate that the GRIP insert indeed encodes a fully translatable protein.

Example 6: Expression of GRIP in prokaryotic cells

To prepare a convenient supply of GRIP protein for biochemical and other studies, the GRIP insert was prepared for cloning into the pGEX4T3 vector (Pharmacia). This vector expresses correctly inserted protein as a fusion with glutathione S-transferase, by means of which it may readily be purified by glutathione sepharose affinity chromatography.

pGRIP.BDI was used as template in three PCR reactions, each designed to tag a particular portion of GRIP sequence with BamHI and EcoRI cloning sites in the appropriate reading frame such that when the resulting fragment was cloned into pGEX4T3, a GST-GRIP fusion protein would be produced.

In one reaction, primers CE65 and CE79 were employed to amplify full length GRIP cDNA, here termed GRIPFL. In a second reaction, primers CE69 and CE70 were used to amplify a portion of GRIP cDNA encompassing the residues coding for the putative SH2 domain (residues 151 to 459 of Figure 5), here termed GRIPSH2. In the third reaction, primers CE71 and CE72 were used to amplify a portion of GRIP cDNA encompassing the residues coding for the insert domain (residues 430 to 825 of Figure 5), here termed GRIPINS.

Each of the three reaction products was digested with BamHI and EcoRI, purified by preparative gel electrophoresis, and ligated into suitably prepared pGEX4T3 to create constructs pGRIPFL.GEX, pGRIPSH2.GEX and pGRIPINS.GEX respectively. Single clones of each of these were analysed by fluorescent dye-terminator sequencing and confirmed to have the expected structure. These plasmids therefore encode GST-GRIP fusion proteins, termed GST-GRIPFL, GST-GRIPSH2 and GST-GRIPINS respectively.

These plasmids were transformed into E. coli strain BL21 (Novagen) and bacterial cultures initiated from single transformant colonies. As a control, parental pGEX4T3 was also transformed into BL21 bacteria and used to initiate cultures: the product of this plasmid is here termed GST. A 200 ml mid-log phase culture of each construct was prepared, and expression of the GST fusion proteins induced by the addition of IPTG to a final concentration of 1 mM in the culture medium. After further growth for approximately 3 hours at 30°C, bacteria were recovered by centrifugation. Bacterial pellets were resuspended in 2.5 ml of either PBS/1% Triton X100 or 25 mM Tris pH8.0/1 mM EDTA/1% Triton X100/0.2% NP40/1 mg/ml lysozyme, each supplemented with Complete protease inhibitors used according to the manufacturer's instruction (Boehringer-Mannheim), and then lysed by sonication. Insoluble matter was removed by centrifugation, and the clarified lysates mixed with 0.67 ml glutathione sepharose 4B previously washed according to the manufacturer's instructions (Pharmacia). These reactions were tumbled for 30 minutes at room temperature, the resin pelleted by centrifugation, and the supernatant discarded. The resin in each sample was then washed with 25 ml 2 mM EDTA/PBS.

In some experiments, the resulting resins were then stored in 2 mM EDTA/PBS at 4°C for short periods of time for later use. These we term 'charged resins', and contain GST or GST-GRIP fusion proteins immobilised onto the glutathione sepharose 4B, and are useful for affinity chromatography protocols involving proteins which associate with GRIP. In other experiments, the GST fusion proteins were eluted from the resin by incubation with 10 mM reduced glutathione/PBS elution buffer, separated from the resin by centrifugation, and stored frozen at -20°C as purified protein. Both charged resins and purified protein were prepared for

GST-GRIPFL, GST-GRIPSH2, GST-GRIPINS and also GST alone (using the parental pGEX4T3 transfected cells).

Samples of the purified proteins were analysed by SDS-PAGE and staining with Coomassie stain. Each preparation contained a major protein band of the size expected for each GST fusion protein, and sometimes a number of faint lower molecular weight bands reflecting partial degradation products.

Example 7: Production of monoclonal antibodies against GRIP

Samples of GST-GRIPFL were used to immunise mice according to a published protocol (Kilpatrick et al, 1997). Hybridoma fusions were prepared, cultured and maintained as described (Kilpatrick et al, 1997). Samples of hybridoma supernatant were used as probe antibody in Western blots of SDS-PAGE gels upon which samples of GST-GRIPFL and GST had been separated. Those cultures whose supernatants showed preferential immunoreactivity for GST-GRIPFL as against GST were selected for further analysis. Cultures whose supernatants additionally showed immunoreactivity for the Xpress-tagged GRIP molecule present in pGRIP-X-transfected COS1 cell lysate as described above were further selected.

From a number of these cultures, clonal populations of hybridomas were prepared by limiting dilution cloning, and the culture supernatants of these clones analysed as described above. A number of clones were chosen, whose supernatants showed immunoreactivity on Western blots for GST-GRIP and Xpress-tagged GRIP, but not for GST. These supernatants therefore contain monoclonal antibodies reactive with GRIP.

One of these monoclonal antibodies, 1-13.4, was employed in another Western blot. In this, cell lysates were prepared according to standard protocols from Jurkat T cells. Samples of these lysates were separated by SDS-PAGE, along with samples of GST-GRIPFL, transferred to nitrocellulose and then probed using the anti-GRIP monoclonal antibody 1-13.4. Bound antibody was visualised as described above. The results are shown in Figure 11. The antibody specifically recognises the GST-GRIPFL protein, and also a single band in the Jurkat lysate at the molecular weight predicted from the GRIP cDNA sequence. No reactivity was observed at molecular weights characteristic of Grb2 or Grap, even though both are expressed in Jurkat cells.

These data illustrate that monoclonal antibodies prepared using GST-GRIPFL may be used for the detection of natively expressed GRIP and recombinant GRIP, and further, that such antibodies may be specific for GRIP, showing no cross-reactivity for the related proteins Grb2 and Grap.

It will be apparent to those skilled in the art that aspects of the present invention such as GST-GRIPFL may be also used in the production, characterisation and purification of other agents which show specific protein binding activity for GRIP or fragments or variants thereof. These may include polyclonal antisera, antibody fragments such as Fab, Fab2, single chain Fv, humanised antibodies, chimaeric antibodies, bispecific antibodies, other antibody derivatives, binding agents derived from polynucleotides such as aptamers, and other similar agents well known to those skilled in the area.

Example 8: GST-GRIP binds to a CD28 phosphopeptide

To provide a convenient assay for the function of GRIP, an assay was prepared in which the binding of GST-GRIPFL, GST-GRIPSH2, GST-GRIPINS and GST to phosphopeptides derived from a portion of the CD28 cytoplasmic domain could be assessed. Two peptides corresponding to the CD28 sequence around tyrosine 173 were chemically synthesised. These had the sequences [biotin]-KLLHSDYMNMTPR ('control phosphopeptide') and [biotin]-KLLHSDpYMNMT

('phosphorylated peptide') where [biotin]-K indicates a lysyl residue bearing a biotin moiety and pY indicates a phosphotyrosine residue. The substantive difference between these sequences lies in the presence or absence of a phosphate group attached to the tyrosine residue.

Nunc Maxisorp microtitre plates were coated with 2 µg/ml of streptavidin (STAR1B; Serotec; Kidlington; UK) in PBS, 100 µl per well, and stored overnight at 4°C. After washing with TBS/0.1% Tween 20, unoccupied protein binding sites on the plate were blocked by incubation with 200 µl per well of a 3% w/v solution of BSA in PBS overnight at 4°C. After further washing as above, wells were exposed to an approximate 5 µM solution of either peptide in PBS, 100 µl per well for approximately 1 hour at room temperature. Plates were washed once again, and then exposed to approximately equal concentration solutions of GST-GRIPFL, GST-GRIPSH2, GST-GRIPINS or GST, 100 µl per well for approximately 1 hour at room temperature. Plates were washed once again, and then exposed to a 1/1000 v/v solution of goat anti-GST antiserum (Pharmacia) in PBS, 100 µl per well for approximately 45 minutes at room temperature. Plates were washed once again, and then exposed to a horseradish peroxidase-conjugated antiserum directed against goat Ig (A5420; Sigma Chemical Co) at a concentration of 1/5000 v/v in PBS, 100 µl per well for approximately 45 minutes at room temperature. Plates were washed for a final time, and the bound peroxidase activity quantitated by use of a chromogenic substrate (Fast OPD; Sigma Chemical Co) according to the manufacturer's instructions. After the chromogenic reaction had proceeded to an appropriate extent, it was terminated by the addition of 3M sulphuric acid, 25 µl per well, and the amount of reaction product quantitated by determining the absorbance at 490 nm.

The results are shown graphically in Figure 12. These data show that none of the GST fusion proteins associate with the unphosphorylated CD28 peptide. GST-GRIPFL and GST-GRIPSH2, but not GST-GRIPINS or GST bind the phosphorylated CD28 peptide, showing a specific interaction between phosphorylated CD28 sequences and GRIP.

These data demonstrate that the GRIP SH2 domain is sufficient to mediate interaction with the region of CD28 sequence centred around phosphorylated tyrosine 173. Furthermore, this interaction is absolutely contingent on phosphorylation of the tyrosine, exactly as observed in the earlier yeast work.

These data also demonstrate that portions of the GRIP sequence may be separated from the whole protein, expressed in a heterologous prokaryotic system, and yet retain biochemical function. From this it is clear that either full length GRIP or various portions or fragments of GRIP, preferably the SH3, SH2 and insert domains, may be used, either alone or in combination with each other or with other proteins (either in their entirety or fragments thereof) to exploit some property of GRIP. For some applications, fragments may be superior to the full-length GRIP protein. For example, a polypeptide including the GRIP SH2 domain, may be used to modulate the interaction of phosphorylated CD28 or other phosphoproteins with natively expressed GRIP. In another example, sufficient fragment of GRIP to bind a given partner protein might be combined with a detectable marker in order to facilitate detection of GRIP-binding partner proteins, or with an enzyme such as a protease in order to target the enzyme activity to the GRIP-binding partner protein. Such combinations and fusions may be accomplished by genetic engineering, in which chimaeric genes encoding the desired polypeptide are constructed, or by crosslinking preformed proteins, or by other similar approaches well known to those skilled in the art. Furthermore, it is also contemplated that deletions, additions or mutations may be made to the GRIP fragments in order to optimise their properties for the desired purpose.

It will be readily apparent to one skilled in the art that such simple biochemically defined assays may be easily adapted and utilised to screen for agents that specifically modulate the interaction between full length GRIP or fragments of GRIP and CD28 or other proteins or peptides derived therefrom.

Furthermore, these data also suggest that agents like the CD28 phosphorylated peptide will act as modulators of GRIP SH2 domain function. In this particular instance, the CD28 phosphorylated peptide will act as an antagonist of GRIP binding to full length CD28, by means of its affinity for the GRIP SH2 domain. Modifications may be made to this or similar peptides to add or modify desirable properties such as cell permeability, oral bioavailability, stability, affinity, specificity; or to eliminate or ameliorate undesirable properties; without departing from the scope of the invention.

Example 9: GRIP binds specifically to CD28

To elucidate further the specificity of the interaction between GRIP and CD28, experiments were conducted in the yeast two hybrid system to compare the interactions between GRIP and CD28 or CD3 ζ . Like CD28, CD3 ζ is another T cell molecule which is phosphorylated by Lck and also forms the site of attachment for SH2 containing signalling proteins.

The BamHI-EcoRI fragment of full length GRIP used in the construction of pGRIPFL.GEX was also ligated into suitably prepared pACT2/BamHI to generate a construct termed pGRIPFL.AD which encodes a GAL4AD-GRIPFL fusion protein. Individual clones of this construct were sequenced to confirm their structure.

pGRIPFL.AD plasmid DNA was cotransfected into Ylck4.1 yeast (Fuller et al, 1998) with pCD28Y.BD plasmid DNA or pAS2/TCR ζ plasmid DNA (Fuller et al, 1998). This latter plasmid, a kind gift of Dr MJ Sims, GlaxoWellcome UK, contains an in-frame fusion of GAL4BD to the cytoplasmic domain of human CD3 ζ . Transformants were grown on either Met⁺ or Met⁻ media, to either repress or induce expression of the Lck protein tyrosine kinase. Resulting colonies were analysed for LacZ activity as described (Fuller et al, 1998). The results are shown in the table below, scored according to the amount of LacZ activity seen. - indicates no LacZ activity, + indicates trace amounts of LacZ activity, ++, +++ and ++++ indicate progressively stronger amounts of LacZ activity.

GAL4BD plasmid	Lck expression	
	Induced	Repressed
pCD28Y.BD	++++	-
pAS2/TCR ζ	-	-

These data indicate that full length GRIP interacts strongly with CD28 cytoplasmic domain only in the presence of Lck which is capable of phosphorylating the residue corresponding to tyrosine 173. However, GRIP does not associate with the cytoplasmic domain of CD3 ζ , regardless of the presence or absence of Lck, which is capable of phosphorylating a number of tyrosine residues in CD3 ζ . In a parallel control preparation, Ylck4.1 yeast containing both the pAS2/TCR ζ plasmid and also the pACT2/ZAP70SH2 plasmid (Fuller et al, 1998) were analysed: ++++ LacZ activity was obtained upon induction of Lck, - LacZ activity when the Lck was repressed.

These data demonstrate that GRIP interacts specifically with the CD28 cytoplasmic domain, but not generally with other tyrosine-bearing signalling domains which are also capable of being phosphorylated by Lck. These data suggest that agents which modulate the function of GRIP are likely to have specific effects on the CD28 signalling machinery. This specificity greatly increases the therapeutic utility of the present invention, as it provides a route for obtaining a desired effect on the B7 costimulatory system, without adversely affecting other T cell control systems.

Although we have only examined T cells as an example of a cell where both CD28 and GRIP are expressed, there are preliminary reports of a small number of other cell types in which CD28 mRNA or protein may be found, including mast cells and plasma cells. Such cell types

may also represent targets for interventions aimed at modulating a CD28-dependent pathway by manipulating the function of GRIP.

Example 10: Mutational analysis of the GRIP-CD28 interaction

To further delineate the molecular nature of the interaction between GRIP and CD28, a further series of vectors encoding GAL4BD-CD28 fusion proteins were prepared in which one or more residues of the CD28 sequence were mutated in such a fashion as to alter the polypeptide sequence.

Plasmid pCD28F.BD was constructed as described for pCD28Y.BD, except that primer CC209 was used in place of primer CC206, and that the BssHII fragment bearing the CD28 sequence was ligated directly into suitably prepared pAS1CYH2/BssHII. Clones of the correct structure were identified by restriction digestion and sequencing. This plasmid encodes a GAL4BD-CD28 fusion protein in which the tyrosine residue corresponding to CD28 residue 173 is replaced by a phenylalanine residue. This fusion protein is therefore not a substrate for tyrosine phosphorylation at this site.

Plasmid pCD28V.BD was constructed as described for pCD28F.BD, except that primer CE42 was used in place of CC205 and primer CE44 in place of CC206. This plasmid encodes a GAL4BD-CD28 fusion protein in which the methionine residue corresponding to CD28 residue 174 is replaced by a valine residue.

Plasmid pCD28K.BD was constructed as described for pCD28F.BD, except that primer CE42 was used in place of CC205 and primer CE45 in place of CC206. This plasmid encodes a GAL4BD-CD28 fusion protein in which the asparagine residue corresponding to CD28 residue 175 is replaced by a lysine residue.

Plasmid pCD28VK.BD was constructed as described for pCD28F.BD, except that primer CE42 was used in place of CC205 and primer CE46 in place of CC206. This plasmid encodes a GAL4BD-CD28 fusion protein in which the methionine residue corresponding to CD28 residue 174 is replaced by a valine residue and the asparagine residue corresponding to CD28 residue 175 is replaced by a lysine residue.

The various GAL4BD-CD28 fusion protein plasmids were then cotransfected into Ylck4.1 yeast along with either pGRIPFL.AD (encoding a GAL4AD-GRIP fusion protein) or p85SH2C.AD (encoding a GAL4AD-p85 SH2 domain fusion protein). Transformants were grown on either Met⁺ or Met⁻ media, to either repress or induce expression of the Lck protein tyrosine kinase. Resulting colonies were analysed for LacZ activity as described (Fuller et al, 1998). The results are shown in the table below, scored according to the amount of LacZ activity seen. – indicates no LacZ activity, + indicates trace amounts of LacZ activity, ++, +++ and ++++ indicate progressively stronger amounts of LacZ activity.

GAL4AD plasmid	GAL4BD plasmid	Lck expression	
		Induced	Repressed
pGRIPFL.AD	pCD28Y.BD	+++	-
"	pCD28F.BD	-	-
"	pCD28V.BD	++	-
"	pCD28K.BD	-	-
"	pCD28VK.BD	-	-
p85SH2C.AD	pCD28Y.BD	++++	-
"	pCD28F.BD	-	-
"	pCD28V.BD	+++	-
"	pCD28K.BD	+++	-
"	pCD28VK.BD	+++	-

These data demonstrate that the GRIP-CD28 interaction is critically dependent upon both the presence of a tyrosine residue at position 173 and the presence of active Lck. In combination with the result of the experiment described earlier using phosphorylated and unphosphorylated CD28 peptides, these data indicate that GRIP interaction with CD28 is most likely dependent upon the interaction of the GRIP SH2 domain with the sequence surrounding phosphorylated tyrosine 173. Although the CD28 cytoplasmic domain has three other tyrosine residues, each of which potentially may be phosphorylated by Lck, and subsequently potentially bound by GRIP SH2 domain, this experiment shows that only tyrosine 173 is so used. Although GRIP has two SH3 domains potentially capable of interacting with PXXP motifs, of which there are two in the CD28 cytoplasmic domain, any such interaction is insufficient to drive association of GRIP and CD28 in this system.

Furthermore these results also demonstrate that alterations of the amino acid sequence in the near vicinity of the phosphorylated tyrosine residue can dramatically alter the efficiency of association with GRIP. In particular, alterations in the amino acid sequence at position +2 relative to the phosphotyrosine have a particularly strong effect (phosphorylated tyrosine is position 0 in this numbering system). The control experiments using GAL4AD-p85SH2 fusion proteins demonstrate that these alterations do not prevent either phosphorylation of tyrosine 173 or binding by SH2 domains per se: they are having a specific effect upon the association of GAL4AD-GRIP with GAL4BD-CD28.

These findings provide a molecular basis for the specificity of GRIP binding to CD28 demonstrated previously: GRIP association with partner proteins is dependent upon the precise sequence of the partner protein. These data also demonstrate that small changes in this sequence and hence the tertiary structure of the GRIP binding site can be sufficient to completely abrogate association with GRIP. It will be obvious therefore that agents which are capable of inducing such distortions in the three-dimensional structure of the binding site, perhaps by themselves binding to this area, will serve to modulate the association of GRIP with its partner proteins. Indeed, a polypeptide containing a portion of GRIP sufficient to bind its partner proteins would itself serve as such an agent, modulating the interaction of such partner proteins with native GRIP. Such agents may be used to modulate the function of cells in which GRIP or proteins which interact with GRIP are expressed.

Likewise, it is also obvious that mutations within GRIP which serve to distort the tertiary structure of the parts of the GRIP molecule may also modulate or abrogate the association between GRIP and its partner proteins. Furthermore, it also follows that agents which act to so distort the tertiary structure of GRIP will also be capable of such effects. Such agents may be used to modulate the function of cells in which GRIP is expressed.

Furthermore, such simple assays as that presently described may be adapted in fashions obvious to those skilled in the art so as to serve as a screen for the identification of the modulatory agents described above.

Example 11: Cooperativity analysis of the GRIP-CD28 interaction

Although we have demonstrated that any putative interactions between the SH3 domains of GRIP and the PXXP motifs within CD28 are insufficient to mediate association in the yeast system in the absence of a phosphotyrosine-SH2 interaction, this does not exclude the possibility that such an SH3-based interaction may occur, and may contribute to the total binding affinity between GRIP and CD28.

To assess this possibility, we compared the relative efficiencies of binding of GRIP to either wild-type CD28 or a CD28 sequence containing mutations in both of the PXXP motifs. It is formally possible that these mutations may alter either the efficiency with which tyrosine 173 may be phosphorylated, or reduce the general accessibility of the phosphorylated motif to

binding by SH2 domains. To take these possibilities into account, we also examined the relative efficiency of binding of a control CD28 binding partner which binds to the same phosphorylated motif. This control partner consists of the C terminal SH2 domain of human p85 α . Since it does not have any SH3 domains, it would not be predicted to have the scope for specific interaction with the CD28 PXXP residues, and any perturbation of its binding by the mutations would most likely be due to such effects as described above. Any relative excess perturbation of GRIP binding by these mutations, over and above that seen for the control protein, is indicative of an active role played by the PXXP motifs, most likely through interaction with the GRIP SH3 domains.

These experiments were performed in the yeast two hybrid system. First, a PCR fragment containing the complete intracellular domain of human CD28 was constructed by PCR as for pCD28Y.BD, except that primer CC1208 was used in place of CC206 and CC1211 in place of CC207. The resulting PCR product was digested with BssHII and NotI, and cloned into a suitably prepared variant of pGEX4T3 that possesses Ascl and NotI cloning sites. This vector was simply used to facilitate cloning and sequencing of the PCR product – any similar vector with single instances of these restriction sites could be used in the same role. Ascl and BssHII restriction sites have compatible overhangs, such that a fragment cleaved with BssHII may be ligated into an Ascl site, and subsequently liberated from this site by digestion with BssHII.

A clone of the correct structure was identified by fluorescent dye-terminator sequencing and the insert removed by digestion with BssHII and NotI. This fragment was then ligated into suitably prepared pAS1CYH2/BssHII and a plasmid clone of the correct structure identified by sequencing. The resulting plasmid, pCD28PP.BD, encodes a GAL4BD-CD28 fusion protein in which the proline residues normally found at positions 178, 181, 190 and 193 of the mature human CD28 molecule are replaced by alanine residues.

Samples of plasmids pCD28Y.BD, pCD28F.BD and pCD28PP.BD were individually cotransfected into Y4.11ck yeast with samples of either pGRIPFL.AD or p85SH2C.AD plasmid. pCD28F.BD, which encodes a GAL4BD-CD28 fusion protein in which tyrosine 173 equivalent residue is replaced by phenylalanine. This fusion protein is therefore incapable of being phosphorylated at this site, and hence of supporting SH2 domain binding. The LacZ signals produced by the various GAL4BD fusion proteins in combination with this GAL4AD-CD28 fusion protein therefore define the background level of LacZ activity in the experiment.

Transformants were selected on media deficient in tryptophan and leucine as described (Fuller et al, 1998), and single colonies grown up in liquid culture in Met- media to induce the Lck gene. To analyse the amount of LacZ activity induced by the interaction of the two hybrid fusion partners, we employed a quantitative liquid assay similar to that described by Harshman et al (1988). 1 ml samples of mid-log phase cultures were harvested by centrifugation and resuspended in 100 mM Tris pH7.5/0.05% Triton X100, 200 μ l per sample. The yeast cells were lysed by two cycles of rapid freezing in liquid nitrogen followed by rapid thawing in a 37°C water bath. For each sample, a tube containing 600 μ l of chromogen solution was prepared. Chromogen solution is a mixture of Z-buffer, ONPG solution and 2-mercaptoethanol mixed in the volume ratio 500:100:1.644. Z-buffer is 60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 0.1 mM MgSO₄, pH7.0. ONPG solution is 4 mg/ml w/v o-nitrophenyl- β -D-galactopyranoside (Sigma Chemical Co) prepared in 100 mM sodium phosphate buffer pH7.5. The lysate samples were vortexed vigorously, and a fixed volume V transferred to the prepared tubes containing the chromogen solution. These were rapidly vortexed and transferred to a 37°C water bath where they were incubated for a time T before the reaction was terminated by the addition of 0.25V 1M Na₂CO₃ solution. Each tube was then centrifuged and the absorbance of a sample of the supernatant measured at 420 nm, giving the value A. To allow normalisation of the amount of LacZ activity according to the number of cells in each culture, a volume of the lysate W μ l was diluted in 1 ml water and the absorbance measured at 600 nm, giving value B. The activity U of LacZ was then obtained, calibrated in arbitrary units, according to the formula $U = 1000 A W / B V T$.

The findings from a representative experiment were as follows:

GAL4AD fusion	LacZ activity produced by interaction with GAL4BD fusion (units)		
	pCD28Y.BD	pCD28F.BD	pCD28PP.BD
pGRIPFL.AD	0.322	0.005	0.040
p85SH2C.AD	0.374	0.005	0.225

These data show that for GRIP, the amount of LacZ activity produced by the association with the PXXP mutant CD28 is only 12.4% of that produced by association with the wild-type CD28. For the p85SH2 domain, the proportion is 60%. These results may be interpreted as follows. While the mutations in the CD28PP construct do affect the efficiency of interaction with p85SH2, as assessed by LacZ activity induction, the same mutations affect the interaction with GRIP to a much greater degree, indicative of a contribution of the PXXP motifs to the total interaction efficiency. These results demonstrate that one or both of the CD28 PXXP motifs are required for optimal binding efficiency to GRIP, most likely through their association with one or both of GRIP's SH3 domains. In combination with our earlier results, these data demonstrate that such associations are neither necessary nor sufficient for GRIP binding to CD28, but form a substantial contributory element.

It will be obvious therefore that agents which perturb the interactions between either or both of the GRIP SH3 domains and binding partner proteins like CD28, while they may not necessarily abrogate GRIP binding, may nevertheless substantially modulate the total binding efficiency. Such modulation may dramatically alter the nature or magnitude of the function performed by GRIP in any particular system, for example by altering the half-life of a complex between GRIP and a partner protein, or in the case where one or more other proteins compete with GRIP for a mutually exclusive binding site on a partner protein, by altering the relative balance between GRIP and these other proteins in binding to the partner protein. Such modulation may be therapeutically desirable in conditions where GRIP is over-active, or insufficiently active or displays an inappropriate type of activity, or where competing proteins display undesired activity. It will also be obvious that the type of system described here may readily be adapted and varied by one ordinarily skilled in the art in order to search for such agents by screening.

Example 12: GRIP is recruited to activated CD28 receptor in vivo

To investigate whether GRIP forms a physiological part of the CD28 signalling complex, CD28 receptor was activated by cross-linking with an activating antibody, immunoprecipitated, and the resulting co-precipitating proteins analysed by Western blotting.

16×10^7 Jurkat T cells were washed in culture medium lacking foetal calf serum (SFM), pelleted by centrifugation, resuspended in 4 ml of SFM, and divided into 2 equal volume aliquots. These were prewarmed to 37°C for 10 minutes and then 20 µg of goat antibody to mouse Ig (cat M2650; Sigma Chemical Co) were added to each aliquot.

After a further two minutes incubation at 37°C, one aliquot of cells, labelled t=0, was lysed by the addition of 2 ml of 2 x RIPA (2% NP40, 1% sodium deoxycholate, 0.2% SDS in PBS + protease/phosphatase inhibitors: Complete cocktail; Boehringer-Mannheim cat ; made up to twice normal concentration + 2 mM sodium orthovanadate)) and incubated on ice for 15 minutes. After this lysis step, 5.6 µg of anti-CD28 antibody clone 9.3 (see for example Bjorn Dahl et al, 1989) were added. To the second aliquot of cells, labelled t=4, 5.6 µg of the anti-CD28 antibody were added and the cells incubated at 37°C for 4 minutes. They were then lysed as for the first aliquot.

Both samples were then centrifuged to remove insoluble matter, and then tumbled with 80 μ l per sample of Protina/G agarose (Pierce) overnight at 4°C. After centrifugation, the supernatants were discarded and the resins in each sample were then washed extensively in 1 x RIPA (1% NP40, 0.5% sodium deoxycholate, 0.1% SDS in PBS; + protease/phosphatase inhibitors: Complete cocktail made at normal concentration + 1 mM sodium orthovanadate). Bound proteins were then eluted from the resins by boiling for 5 minutes in SDS-PAGE sample buffer. Stripped resins were pelleted by centrifugation, and samples of the supernatant containing eluted proteins analysed by SDS-PAGE and Western blotting by standard means.

In one such experiment, a Western blot of these samples was performed using monoclonal antibody to GRIP clone 1-13.4. In addition to samples of the t=0 and t=4 preparations, the blot also contained samples of Jurkat lysate which had been prepared simply by lysing Jurkat T cells in 2 x RIPA and immediately adding SDS-PAGE sample buffer, without any immunoprecipitation manipulations. This sample provides an internal control for the Western blotting part of the experiment.

The results are shown in Figure 13. In addition to non-specific signal derived from the cross-reaction of the HRP-anti mouse Ig secondary antibody with the murine anti-CD28 used for the immunoprecipitation, there is also a specific band, at the same molecular weight as GRIP, which appears in the t=4 sample and not in the t=0 sample. These data demonstrate that GRIP is indeed specifically recruited to the activated CD28 receptor.

This assay may also form the basis of a diagnostic kit for the determination of whether CD28 expressed on a lymphocyte surface has been recently activated, as might occur at a higher level than normal in autoimmune diseases, or at a lower level than normal in cancer or other immunosuppressive conditions. In such a kit, patient T lymphocytes would be purified by standard methods, lysed using the lysis buffer described above, and then the CD28 molecules immunoprecipitated as described above. Antibodies against GRIP antibody would then be used to determine whether the precipitated CD28 was associated with GRIP.

Furthermore, these data also demonstrate that GRIP is recruited to CD28 when the receptor is activated. Since therapies and manipulations which antagonise the CD28 signal are efficacious in autoimmune diseases, as demonstrated by in vivo experiments in models of rheumatoid arthritis, lupus, graft-versus-host-disease, transplant rejection, inflammatory bowel disease, multiple sclerosis, psoriasis, allergic asthma and contact dermatitis for example, it is to be expected that interventions which inhibit the function of GRIP will be similarly useful. Moreover, in diseases like cancer and immunosuppression where T cells are insufficiently activating, the provision of a CD28 signal, perhaps through the use of stimulating antibodies, has beneficial effects. In such states, interventions which promote the function of GRIP, perhaps by increasing its binding to CD28, or perhaps by increasing the efficiency with which it recruits partner proteins, may also have beneficial effect.

Example 13: GRIP associates with other signalling molecules

We also investigated the association of GRIP with other signalling proteins in order to elucidate the nature and identity of proteins that it might serve to recruit to activated CD28 receptor. 4×10^7 Jurkat cells were washed in culture medium lacking foetal calf serum (SFM), pelleted by centrifugation, resuspended in 1 ml SFM and lysed by the addition of 1 ml of 2 x RIPA. After incubation on ice for approximately 30 minutes, insoluble matter was removed by centrifugation. The resulting clarified lysate was tumbled at 4°C for 1 hour with 2.66 ml of glutathione sepharose 4B resin (Pharmacia) (previously washed in PBS according to the manufacturer's instructions and then equilibrated into 1x RIPA). The resin and any bound protein were removed by centrifugation, and the remaining supernatant (precleared lysate) divided into four equal volume aliquots.

Samples of glutathione sepharose 4B resin charged with either GST-GRIPFL, GST-GRIPSH2, GST-GRIPINS or GST were prepared as described above. Each sample of charged resin was mixed with one aliquot of preclarified lysate and tumbled overnight at 4°C. The resin was pelleted by centrifugation and the supernatants discarded. After extensive washing of the resin with 1 x RIPA, bound proteins were eluted by boiling the resin in 100 µl of SDS-PAGE sample buffer for 5 minutes. Stripped resins were pelleted by centrifugation, and samples of the supernatant containing eluted proteins analysed by SDS-PAGE and Western blotting by standard means.

In one such experiment, the eluted proteins were analysed for the presence of Sos2 using a Sos2-specific antiserum (Santa Cruz catalogue number SC258). A band of the expected molecular weight was observed only in the sample where Jurkat lysate had been exposed to GST-GRIPFL (Figure 14). These data indicate that the full length GRIP protein has the potential to specifically associate with signalling proteins such as Sos2, and therefore to recruit these proteins to activated CD28 receptor. The absence of association between GST-GRIPSH2 or GST-GRIPINS and Sos2 implies that it is most likely one or both of the GRIP SH3 domains, present only in GST-GRIPFL, which mediates the particular interaction with Sos2. Similarly, GRIP may associate with other proteins by means of either or both of the SH3 domains, the SH2 domain or the insert domain, or combinations of these or fragments thereof.

Example 14: Development of a yeast assay for proteins that associate with GRIP

To facilitate the identification of proteins which associate with GRIP, a yeast two hybrid assay was developed. Plasmid pGRIP.BDI (see above) was linearised by digestion with XbaI and transfected into *Saccharomyces cerevisiae* Y190 (Harper et al, 1993). Transformed clones were identified by growth on tryptophan-deficient media, and single clonal colonies isolated by two rounds of streaking out of single colonies. Six such clones were grown up and chromosomal DNA purified as described (Fuller et al, 1998).

Two oligonucleotide primers were designed, one selectively hybridising to sequence encoding the GAL4BD protein, and the other selectively hybridising to the GRIP coding sequence, on the opposing strand to the first primer. These primers were so chosen that if used in PCR with template DNA derived from pGRIP.BDI, a product band of approximately 520 bp would be obtained. The primers were synthesised and employed in standard PCRs using samples of the yeast clone DNA preparations as template. DNA derived from one clone, termed Y190/pGRIP.BDI, produced the expected band, so this yeast clone was selected for further work.

Oligonucleotide primers CE130 and CASOS2 were designed to amplify cDNA encoding the final 197 amino acid residues of human Sos2 and to provide it with cloning sites such that it could be ligated into pACT2/BamHI to form an in-frame fusion with GAL4AD. This region of Sos2 is rich in Pro-Xxx-Xxx-Pro motifs which may mediate interactions with SH3 containing proteins such as GRIP. The predicted cDNA sequence of Sos2 was produced by making a consensus sequence between the relevant portions of Genbank sequences L20686, AA621168 and H01561. These primers were employed in a RT-PCR using Ready-to-Go reagents according to the manufacturer's instructions (Pharmacia) and 4 µl of total RNA purified from Jurkat cells as described previously. Forty cycles of amplification were applied, and the resulting PCR product purified by means of the Wizard DNA clean up kit (Promega). A sample of this cDNA fragment was then used as template in a second PCR using primers CE130 and CASOS2. The product of this reaction was digested with BamHI and XhoI and ligated into suitably prepared pACT2/BamHI. A number of clones were analysed by restriction digestions diagnostic for the presence of the Sos2 insert, and one positive clone selected for further analysis. This clone was analysed by fluorescent dye-terminator sequencing, and found to have the predicted sequence. This plasmid, pACT2/Sos2, therefore encodes a GAL4AD-Sos2 fusion protein.

Samples of pACT2/Sos2 and parental pACT2/BamHI plasmid DNA were transformed into either yeast Y190/pGRIP.BDI or parental Y190 according to standard protocols. Transformants were selected for growth on media deficient in tryptophan and leucine or leucine only, as appropriate, and subsequently analysed for LacZ activity as described (Fuller et al. 1998). The results are shown in the table below, scored according to the amount of LacZ activity seen. - indicates no LacZ activity, + indicates trace amounts of LacZ activity, ++, +++ and ++++ indicate progressively stronger amounts of LacZ activity.

Plasmid	Yeast strain	
	Y190/pGRIP.BDI	Y190
pACT2/Sos2	++++	-
pACT2	+	Not done

These data indicate that GRIP interacts specifically with the C terminal fragment of Sos2 and that this interaction can be assayed in the yeast two-hybrid format. Since in this yeast system there is no exogenously supplied protein tyrosine kinase, these results also demonstrate that GRIP is capable of forming non-phosphotyrosine-dependent associations with other signalling proteins.

It will be readily apparent to one skilled in the art that similar yeast two hybrid assays may be derived from this work by application of well known molecular biological techniques and applied to the discovery of novel proteins which interact with some part of GRIP protein. It will also be apparent that similar assays may be employed to identify by screening agents which specifically modulate the association of GRIP polypeptides with previously identified protein binding partners. One such agent may be the portion of Sos2 employed above, or peptides or fragments derived therefrom.

Primers

CASOS2: 5' AGGGAATTCCTCGAGTCATTGGGGAGTTTCTGCATTTTCTAG 3'
CC205: 5' CATCGCGCGCAGTAAGAGGAGCAGGCTCCTGCACAGTG 3'
CC206: 5' TCTCGTTGGACCCGGTCTACGTGGAGTCATGTTTCATGTAGTCACTGTGCAGGAGCCTG 3'
CC207: 5' CCGGGTCCAACGAGAAAGCATTACCAGCCCTATGCACCACCTAGAGACTTCGCAGC 3'
CC208: 5' CATCGCGCGCTGCGGCCGCTCAGGAGCGATAGGCTGCGAAGTCTCTAGG 3'
CC209: 5' TCTCGTTGGACCCGGTCTACGTGGAGTCATGTTTCATGAAGTCACTGTGCAGGAGCCTG 3'
CC1208: 5' TCTCGTTGGACCCGGTCTACGTGCAGTCATGTTTCATGTAGTCACTGTGCAGGAGCCTG 3'
CC1211: 5' GCGGGTCCAACGAGAAAGCATTACCAGGCCCTATGCAGCACCTAGAGACTTCGCAGC 3'
CE14: 5' CATCGGATCCGAAGATTTGCCCCATCATG 3'
CE15: 5' CATCGAATTCTCATCGCCTCTGCTGTGC 3'
CE42: 5' CATCGCGCGCAGTAAGAGGAGCAGGCTCCTGCAC 3'
CE44: 5' TCTCGTTGGACCCGGTCTACGTGGAGTCATGTTTCACGTAGTCACTGTGCAGGAGCCTGCTCC 3'
CE45: 5' TCTCGTTGGACCCGGTCTACGTGGAGTCATTTTCATGTAGTCACTGTGCAGGAGCCTGCTCC 3'
CE46: 5' TCTCGTTGGACCCGGTCTACGTGGAGTCATTTTCACGTAGTCACTGTGCAGGAGCCTGCTCC 3'
CE65: 5' CATCGCGCGCGGATCCAAGCTTATGGAAGCTGTTGCCAAGTTTGATTTG 3'
CE66: 5' CATCGAATTTCGTCGACGCGGCCGCTTATCGGGTCATGGGTGCCACGTA 3'
CE69: 5' CATCGGATCCATAGACATCCAGTTTCCCAAATGG 3'
CE70: 5' CATCGAATTCTTACTGGTCTTCTCGGGTTCTGTC 3'
CE71: 5' CATCGGATCCTTCCTTAGAGACAGAACCCGAGAA 3'
CE72: 5' CATCGAATTCTTACCACCGCACTCGCCCTGCCGCCTG 3'
CE79: 5' CATCGCGGCCGCGTCGACGAATTCTTATCGGGTCATGGGTGCCACGTA 3'
CE102: 5' ACCACAGTCCATGCCATCAC 3'
CE103: 5' TCCACCACCCTGTTGCTGTA 3'
CE106: 5' GATCTGTACGACGATGACGATAAGTCTAGAG 3'
CE107: 5' GATCCTCTAGACTTATCGTCATCGTCGTACA 3'
CE108: 5' GATGGAATTCAGCACACAGGACCTCACCATGGGGGGTTCTCATCATC 3'
CE109: 5' GATGGAATTCTTATCGGGTCATGGGTGC 3'
CE110: 5' GATGGAATTCAGCACACAGGACCTCACCATGTACCCATACGATGTTCCAGATTA
CGCTGAAGCTGTTGCCAAGTTTG 3'
CE130: 5' ATAAGATGGCGCGCGGATCCTTACATAAACTAAGTGAAGAG 3'

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Claims:

1. A polypeptide comprising the amino acid sequence shown in Figure 5 or any fragment thereof containing at least the amino acid residues encoded by nucleotide residues 151-459 or any polypeptide having substantially the same sequence and capable of binding to human CD28.
2. A polypeptide according to claim 1 wherein the polypeptide has a sequence that is at least 80% homologous to that of Figure 5.
3. A polypeptide according to claim 1 wherein the polypeptide has sequence that is at least 95% homologous.
4. A polypeptide according to claim 1 wherein the polypeptide has sequence that is at least 99% homologous.
5. A polypeptide according to any of the above claims which is capable of binding to CD28 at or near phosphorylated tyrosine 173.
6. A polypeptide according to any of the above claims which is attached to a carrier molecule.
7. A method of preventing a polypeptide according to any of the above claims binding to human CD28 comprising the use of a compound which is capable of inhibiting such binding.
8. A method according to claim 7 wherein the compound which is capable of inhibiting binding between CD28 and said polypeptide is selected from an antibody, antibody derivatives, peptides, phosphorylated peptides or aptamers.
9. A method of identifying a compound that inhibits binding between CD28 and a polypeptide according to any of claims 1 to 6, which comprises use of a polypeptide according to any of claims 1 to 6 to screen for compounds that bind to said polypeptide.
10. A method of identifying a compound that inhibits binding between CD28 and a polypeptide according to any of claims 1 to 6 which comprises use of CD28 to screen for compounds that will bind to CD28 at or near phosphorylated tyrosine 173.
11. A method of treating a human patient with a disorder involving CD28-expressing cells comprising administering to the patient a compound that inhibits the binding of a polypeptide according to any of claims 1 to 6 to CD28.
12. A method according to claim 11 wherein said disorder is an autoimmune disorder or cancer.
13. A DNA sequence encoding a polypeptide according to any of claims 1 to 6.

Figur 1

 Polylinker of pYTH9/BssHII

<i>SalI</i>				<i>NcoI</i>		<i>ClaI</i>		<i>EcoRI</i>		<i>PstI</i>		<i>BssHII</i>				
GCT-AGG-TCG-ACG-GCC-ATG-GTA-TCG-ATG-AAT-TCC-TGC-AGC-CCG-GCG-CGC-TCT-																
A	R	S	T	A	M	V	S	M	N	S	C	S	P	A	R	S

<i>SpeI</i>				<i>NotI</i>					
GGA-TCT-ACT-AGT-GCG-GCC-GCC-ACC-GCG-GTG									
G	S	T	S	A	A	A	T	A	V

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Figure 2

Polylink r of pAS1CYH2/BssHII

NdeI *SfiI* *NcoI* *BssHII* *BamHI* *Sall*
CAT-ATG-GCC-ATG-GAG-GCC-CCG-GCG-CGC-TCT-GGA-TCC-GTC-GAC-CTG-CAG-CCA-
H M A M E A P A R S G S V D L Q P

AGC-TAA
S Stop

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Figure 3

Polylinker of pACT2/BamHI

NdeI *SfiI* *NcoI* *SmaI* *BamHI* *EcoRI*
CAT-ATG-GCC-ATG-GAG-GCC-CCG-GGG-ATC-GGA-TCC-GAT-CCG-AAT-TCG-AGC-TCG
H M A M E A P G I G S D P N S S S

XhoI
AGA-GAT-CTA-TGA
R D L Stop

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Figur 4

Construction of artificial gene encoding the CD28 cytoplasmic domain in a form suitable for expression in the yeast two hybrid vectors. The domain is built up from overlapping primers (underlined) according to the scheme below:

```

      BssHII
      catcgcgcgcAGTAAGAGGAGCAGGCTCCTGCACAGTGACTACATGAACATGACTCCACG
1  -----+-----+-----+-----+ 60
      gtagcgcgcgTCATTCTCCTCGTCCGAGGACGTGTCACTGATGTACTGTACTGAGGTGC

      A  R  S  K  R  S  R  L  L  H  S  D  Y  M  N  M  T  P  R  -

      TAGACCGGGTCCAACGAGAAAGCATTACCAGCCCTATGCACCACCTAGAGACTTCGCAGC
61 -----+-----+-----+-----+ 120
      ATCTGGCCCAGGTTGCTCTTTCGTAATGGTCGGGATACGTGGTGGATCTCTGAAGCGTCG

      R  P  G  P  T  R  K  H  Y  Q  P  Y  A  P  P  R  D  F  A  A  -

      NotI   BssHII
      CTATCGCTCCTGAgcgccgcagcgcgcgatg
121 -----+-----+-----+ 152
      GATAGCGAGGACTcgccggcgctcgcgcgctac

      Y  R  S  *
  
```

Two GC-rich stretches in the sequence have been recoded using the redundancy of the genetic code to give a more balanced base composition while retaining the same amino acid coding sequence. The construct includes restriction sites (bold): BssHII and NotI. The sequence includes only 1 extra residue not part of CD28 - the N-terminal Ala (double-underlined).

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Figure 5

Coding sequence of human GRIP: cDNA and deduced amino acid sequence

```

1  ATGGAAGCTGTTGCCAAGTTTGATTTCAGTCTCAGGTGAGGATGAACTGAGCTTTCAC 60
   -----+-----+-----+-----+-----+
   TACCTTCGACAACGGTTCAAACCTAAAGTGACGAAGTCCACTCCTACTTGACTCGAAAGTG
   M E A V A K F D F T A S G E D E L S F H
61  ACTGGAGATGTTTTGAAGATTTAAGTAACCAAGAGGAGTGGTTTAAGGCGGAGCTTGGG 120
   -----+-----+-----+-----+-----+
   TGACCTCTACAAAACCTCTAAATTCATTGGTTCTCCTCACCAATTCGCGCTCGAACCC
   T G D V L K I L S N Q E E W F K A E L G
121 AGCCAGGAAGGATATGTGCCCAAGAATTTATAGACATCCAGTTTCCCAATGGTTTCAC 180
   -----+-----+-----+-----+-----+
   TCGGTCCTTCTATACACGGGTTCTTAAAGTATCTGTAGGTCAAAGGGTTTACCAAAGTG
   S Q E G Y V P K N F I D I Q F P K W F H
181 GAAGGCCTCTCTCGACACCAGGCAGAGAACCTTACTCATGGGCAAGGAGGTTGGCTTCTTC 240
   -----+-----+-----+-----+-----+
   CTTCGGGAGAGAGCTGTGGTCCGTCTCTTGAATGAGTACCCGTTCTCTCAACCGAAGAAG
   E G L S R H Q A E N L L M G K E V G F F
241 ATCATCCGGGCCAGCCAGAGCTCCCCAGGGGACTTCTCCATCTCTGTCAGGCATGAGGAT 300
   -----+-----+-----+-----+-----+
   TAGTAGGCCCGGTTCGGTCTCGAGGGGTCCCTGAAGAGGTAGAGACAGTCCGTACTCCTA
   I I R A S Q S S P G D F S I S V R H E D
301 GACGTTCAACACTTCAAGGTCATGCGAGACAACAAGGTAATTACTTTCTGTGGACTGAG 360
   -----+-----+-----+-----+-----+
   CTGCAAGTTGTGAAGTTCAGTACGCTCTGTTGTTCCTTAATGAAAGACACCTGACTC
   D V Q H F K V M R D N K G N Y F L W T E
361 AAGTTTCCTTCCCTAAATAAGCTGGTAGACTACTACAGGACAAATTCCTCTCCAGACAG 420
   -----+-----+-----+-----+-----+
   TTCAAAGGAAGGGATTATTCGACCATCTGATGATGTCTGTTAAGGTAGAGGTCTGTC
   K F P S L N K L V D Y Y R T N S I S R Q
421 AAGCAGATCTTCTTAGAGACAGAACCCGAGAAGACCAGGGTCACCGGGGCAACAGCCTG 480
   -----+-----+-----+-----+-----+
   TTCGTCTAGAAGGAATCTCTGTCTTGGGCTCTTCTGGTCCCAGTGGCCCCGTTGTCGGAC
   K Q I F L R D R T R E D Q G H R G N S L
481 GACCGGAGGTCCCAGGGAGGCCACACCTCAGTGGGGCTGTGGGAGAAGAAATCCGACCT 540
   -----+-----+-----+-----+-----+
   CTGGCCTCCAGGGTCCCTCCGGGTGTGGAGTCACCCGACACCTCTTCTTTAGGCTGGA
   D R R S Q G G P H L S G A V G E E I R P
541 TCGATGAACCGGAAGCTGTCCGATCACCCCCGACCTTCCCCTGCAGCAGCACCAGCAC 600
   -----+-----+-----+-----+-----+
   AGCTACTTGGCCTTCGACAGCCTAGTGGGGGGCTGGGAAGGGGACGTCGTCGTGGTCGTG
   S M N R K L S D H P P T L P L Q Q H Q H
601 CAGCCACAGCCTCCGCAATATGCCCCAGCGCCCCAGCAGCTGCAGCAGCCCCACAGCAG 660
   -----+-----+-----+-----+-----+
   GTCGGTGTCGGAGGCGTTATACGGGGTCGCGGGGTCGTCGACGTCGTCGGGGGTGTCGTC
   Q P Q P P Q Y A P A P Q Q L Q Q P P Q Q

```

Figur 5 (continu d)

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661 CGATATCTGCAGCACCACCATTTCACCAGGAACGCCGAGGAGGCAGCCTTGACATAAAT 720
 -----+-----+-----+-----+-----+-----+-----+-----
 GCTATAGACGTCGTGGTGGTAAGGTGGTCCTTGCGGCTCCTCCGTCGGAACGTATTTA

 R Y L Q H H H F H Q E R R G G S L D I N
 721 GATGGGCATTGTGGCACC GGCTTGGGCAGTGAATGAATGCGGCCCTCATGCATCGGAGA 780
 -----+-----+-----+-----+-----+-----+-----+-----
 CTACCCGTAACACCGTGGCCGAACCCGTCACCTTACTTACGCCGGGAGTACGTAGCCTCT

 D G H C G T G L G S E M N A A L M H R R
 781 CACACAGACCCAGTGCAGCTCCAGGCGGCAGGGCGAGTGCGGTGGGCCCGGGCGCTGTAT 840
 -----+-----+-----+-----+-----+-----+-----+-----
 GTGTGTCTGGGTCACGTCGAGGTCCGCCGTCCCGCTCACGCCACCGGGCCCGCGACATA

 H T D P V Q L Q A A G R V R W A R A L Y
 841 GACTTTGAGGCCCTGGAGGATGACGAGCTGGGGTTCCACAGCGGGGAGGTGGTGGAGGTC 900
 -----+-----+-----+-----+-----+-----+-----+-----
 CTGAAACTCCGGGACCTCCTACTGCTCGACCCCAAGGTGTCGCCCTCCACCACCTCCAG

 D F E A L E D D E L G F H S G E V V E V
 901 CTGGATAGCTCCAACCCATCCTGGTGGACCGGCCGCTGCACAACAAGCTGGGCTTCTTC 960
 -----+-----+-----+-----+-----+-----+-----+-----
 GACCTATCGAGGTGGGTAGGACCACCTGGCCGGCGGACGTGTTGTTGACCCGAAGAAG

 L D S S N P S W W T G R L H N K L G F F
 961 CCTGCCAACTACGTGGCACCCATGACCCGATAA 993
 -----+-----+-----+-----+-----+-----+-----+-----
 GGACGGTTGATGCACCGTGGGTACTGGGCTATT

 P A N Y V A P M T R *

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Figure 6

Tissue expression of GRIP mRNA.

Figure 6



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Figure 7

Expression of GRIP mRNA in cell lines

Figure 7

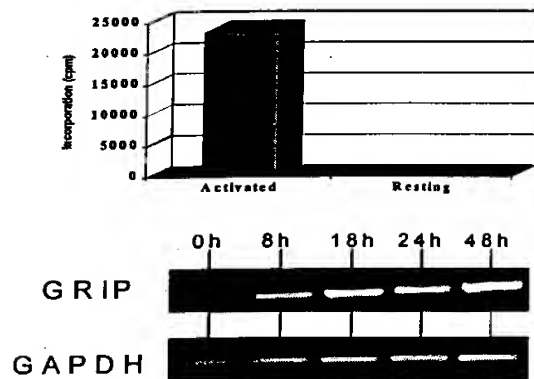


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Figure 8

Modulation of GRIP mRNA levels by cell activation

Figure 8



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Figure 9

Junction sequences in pGRIPFL.FIX. GRIP sequences are underlined.

```
1  AGCTTACCATGGGGGGTTCTCATCATCATCATCATGGTATGGCTAGCATGACTGGTG 60
   -----+-----+-----+-----+-----+
   TCGAATGGTACCCCCAAGAGTAGTAGTAGTAGTAGTACCATACCGATCGTACTGACCAC
       M G G S H H H H H H G M A S M T G G

61  GACAGCAAATGGGTCGGGATCTGTACGACGATGACGATAAGTCTAGAGGATCCAAGCTTA 120
   -----+-----+-----+-----+-----+
   CTGTCGTTTACCCAGCCCTAGACATGCTGCTACTGCTATTTCAGATCTCCTAGGTTTCAAT
       Q Q M G R D L Y D D D D K S R G S K L M

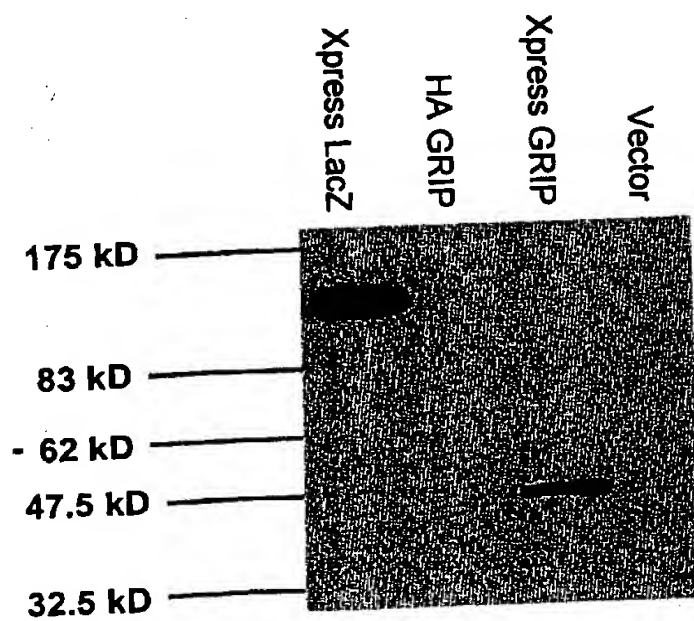
121  TGGAAAGCTGTTGCCAAGTTTGATTTCACTGCTTCAGGTGAGGATGAAGTGAAGCTTTCACA 180
   -----+-----+-----+-----+-----+
   ACCTTCGACAACGGTTCAAACATAAGTGACGAAGTCCACTCCTACTTGACTCGAAAGTGT
       E A V A K F D F T A S G E D E L S F H T

181  CTGGAGATGTTTTGAAGATTTTAAGTAACCAAGAGGAGTGGTTTAAGGCGGAGCTTGGGA 240
   -----+-----+-----+-----+-----+
   GACCTCTACAAAATTCTAAAATTCATTGGTTCTCCTCACCAAAATCCGCCTCGAACCCCT
       G D V L K I L S N Q E E W F K A E L G
```

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Figure 10

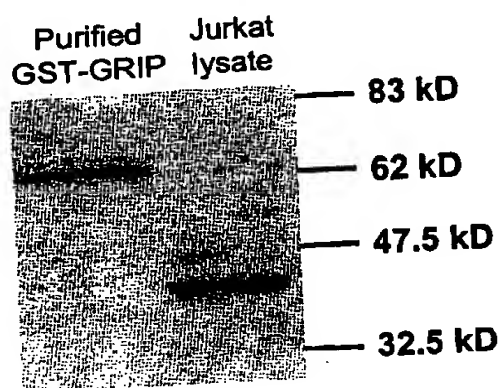
Expression of Xpress-tagged GRIP in eukaryotic cells.



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Figure 11

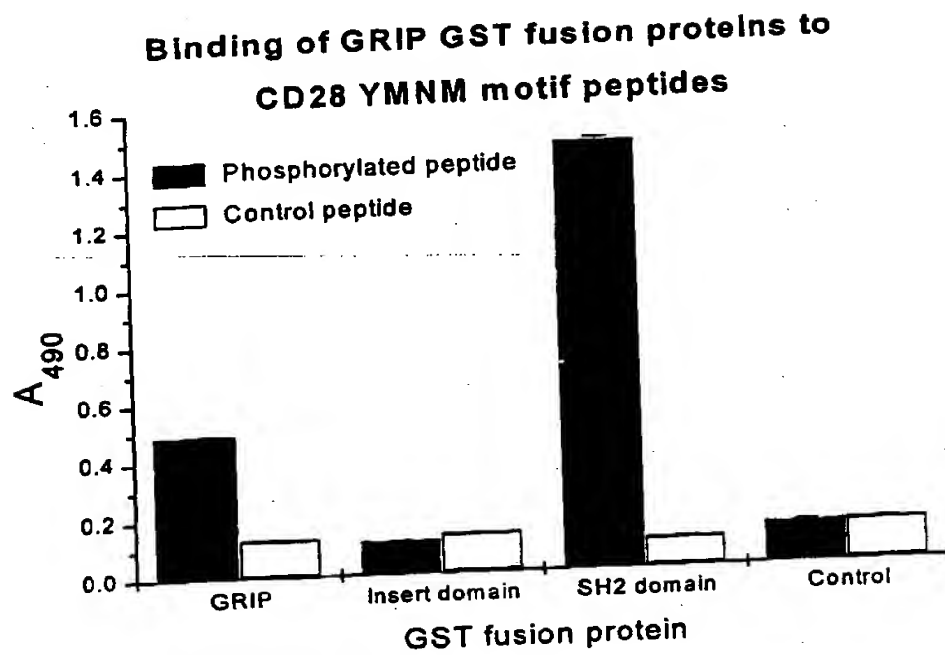
Recognition of natively expressed GRIP from Jurkat cells by anti-GRIP monoclonal antibody 1-13.4.



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Figure 12

Binding of GST-GRIP fusion proteins to CD28 peptides.



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Figur 13

Association of GRIP with activated CD28 receptor.

CD28 crosslink
t=4 t=0

Jurkat
lysate



GRIP

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Enx & Wellcome plc
18/8/99

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